Recommendations on terminology and experimental best practice associated with plant nitric oxide research

DR JAGADIS GUPTA KAPUGANTI (Orcid ID : 0000-0002-7090-5097)

DR RENAUD BROUQUISSE (Orcid ID : 0000-0002-7818-9662)

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Kapuganti Jagadis Gupta[1]*, John T. Hancock[2], Marek Petrivalsky[3], Zsuzsanna Kolbert[4], Christian Lindermayr[5], Jorg Durner[5], Juan B. Barroso[6], José M. Palma[7], Renaud Brouquisse[8], David Wendehenne[9], Francisco J. Corpas[10], Gary J. Loake[11]*

[1] National Institute of Plant Genome Research Aruna Asaf Ali Marg, -110067, New Delhi, India

[2] Department of Applied Sciences, University of the West of England, Bristol BS16 1QY, UK

[3] Department of Biochemistry, Faculty of Science, Palacký University, Šlechtitelů 27, CZ-783 71 Olomouc, Czech Republic

[4] Department of Plant Biology, University of Szeged, Szeged, 6726, Hungary

[5] Institute of Biochemical Plant Pathology, Helmholtz Zentrum München – German Research Center for Environmental Health, München/Neuherberg, 85764, Germany

[6] Group of Biochemistry and Cell Signaling in Nitric Oxide, Department of Experimental Biology, Center for Advanced Studies in Olive Grove and Olive Oils, Faculty of Experimental Sciences, Campus Universitario 'Las Lagunillas' s/n, University of Jaén, 23071, Jaén, Spain

[7] Group of Antioxidants, Free Radicals and Nitric Oxide in Biotechnology, Food and Agriculture, Department of Biochemistry and Cell and Molecular Biology of Plants, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas (CSIC), Profesor Albareda 1, 18008 Granada, Spain

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[8] *INRA, CNRS, Université Côte d'Azur, Institut Sophia Agrobiotech, 06903 Sophia Antipolis Cedex, France*

[9] *Agroécologie, AgroSup Dijon, CNRS, INRA, Univ. Bourgogne Franche-Comté, 21000 Dijon, France*

[10] *Group of Antioxidants, Free Radicals and Nitric Oxide in Biotechnology, Food and Agriculture, Department of Biochemistry and Cell and Molecular Biology of Plants, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas (CSIC), Profesor Albareda 1, 18008 Granada, Spain*

[11] *Institute of Molecular Plant Sciences, School of Biological Sciences, University of Edinburgh, Edinburgh, EH9 3JH, UK*

Corresponding authors

Kapuganti Jagadis Gupta
National Institute of Plant Genome Research
Aruna Asaf Ali Marg
110067 New Delhi, India
jgk@nipgr.ac.in
Tel: +91-11-26735111

Gary John Loake
Institute of Molecular Plant Sciences
School of Biological Sciences
University of Edinburgh Edinburgh, UK
gloake@ed.ac.uk
Tel: + 44 (0) 131-650-5332

ORCID IDs: 0000-0002-7090-5097 (KJG); 0000-0002-7989-9180 (GJL); 0000-0003-1579-3632 (MP); 0000-0002-1814-9212 (JC); 0000-0002-7818-9662 (RB); 0000-0002-0213-8850 (JH)

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Summary

Nitric oxide (NO) emerged as a key signal molecule in plants. During the last two decades impressive progress has been made in plant NO research. This small, redox-active molecule is now known to play an important role in plant immunity, stress

responses, environmental interactions, plant growth and development. To more accurately and robustly establish the full spectrum of NO bioactivity in plants, it will be essential to apply methodological best practice. In addition, there are some instances of conflicting nomenclature within the field, which would benefit from standardisation. In this context, we attempt to provide some helpful guidance for best practice associated with NO research and also suggestions for the cognate terminology.

Recommendations on Terminology

S-nitrosylation or *S*-nitrosation?

The reversible, covalent modification of cysteine thiols by NO is termed *S*-nitrosylation. According to the most extensive comprehensive dataset to date, the Arabidopsis proteome contains 1,195 endogenously *S*-nitrosylated peptides belonging to 926 proteins (Hu *et al.,* 2015), which implies the important biological relevance of this post-translational modification (PTM). There has been recent debate in the field over whether *S*-nitrosylation is an appropriate term, as this name implies an enzymatic function. Nitrosylation involves direct addition of NO to a reactant and is derived from chemistry terminology that describes the coordination of NO to a metal centre leading to formation of a metal nitrosyl complex (Ford *et al.,* 2005). These metal nitrosyls can also be formed by other chemical reactions. For instance, a transition metal can react with acidified nitrite *via* a multistep reaction also leading to the formation of a metal nitrosyl complex (Ford *et al.,* 2010). Hence, the more chemically orientated term, S-nitrosation, has been proposed as an alternative expression to that of S-nitrosylation (Heinrich *et al.,* 2013), where the addition of a nitrosonium ion (NO^+) to a nucleophilic group takes place. In the context of proteins, transfer of a NO^+ molecule is a predominant mechanism for oxidation of protein cysteine (Cys) thiols, although the formation and subsequent role of this molecule in *S*-nitrosothiol (SNO) formation also depends on the cellular conditions and the chemical environment surrounding the target Cys embedded within the given protein, respectivley. In this case, we suggest *S*-nitrosation is therefore a more applicable expression for this chemical process.

In mammals, a handful of proteins have been identified, e.g. glyceraldehyde-3-phosphate dehydrogenase (GAPDH), that interact with specific protein targets and transfer their NO moiety, resulting in *S*-nitrosothiol (SNO) formation at a specific Cys residue on the target protein (Kornberg *et al.,* 2010). The proteins driving this PTM can therefore be considered as nitrosylases (Seth *et al.,* 2018). In this case, the term *trans*-nitrosylation might be more appropriate.

Further, in both mammals and plants, thioredoxin (Trx) enzymes have been shown to directly and selectively remove a SNO from target proteins functioning as de-nitrosylases (Wu *et al.,* 2011; Kneeshaw *et al.,* 2014). Clearly, this process is

mediated by enzyme activity, thus de-nitrosylation rather than de-nitrosation appears a more appropriate term in this context.

Nitrosative stress or nitro-oxidative stress?

The term nitrosative stress refers to a secondary stress condition characterized by a parallel, unregulated increase in the generation of both NO and reactive oxygen species (ROS) (Valderrama *et al.,* 2007). Further, these redox active molecules can react with each other to form additional molecules, such as peroxynitrite ($ONOO^-$), formed by the interaction of NO and superoxide ($O_2^{\cdot-}$). Collectively, these molecules can trigger irreversible damage to different biomolecules, such as proteins, lipids and nucleic acids.

In a similar manner to that of protein carbonylation, which is considered a major hallmark of oxidative stress (Fedorova *et al.,* 2014), an increase in protein tyrosine nitration has been proposed as a plausible marker for nitrosative stress (Corpas *et al.,* 2007). Tyrosine nitration involves an oxidative and a nitrative step and is directly driven by radicals derived from peroxynitrite. Additionally, different antioxidant enzymes, such as catalase, ascorbate peroxidase, monodehydroascorbate reductase and superoxide dismutases, were found to be negatively affected by nitration, further supporting a close relationship between NO and ROS, especially under stress conditions.

Thus, nitro-oxidative stress may be considered a suitable expression to describe cellular events resulting from detrimental accumulation of and interaction between ROS and RNS.

Non-symbiotic hemoglobins or phytoglobins?

Leghemoglobins (Lb) and symbiotic hemoglobins (sym-Hb) are present in either legume species or actinorhizal/non-legume symbiotic nodules, respectively. The presence of hemoglobin-like proteins in organs not association with symbiotic interactions led to the term, non-symbiotic hemoglobins (Hill, 2012; Rubio *et al.,* 2019). These non-symbiotic hemoglobins are thought to function as key scavengers of NO under various environmental and stress conditions (Hill, 2012). Therefore, these proteins may play an important role in NO homeostasis within various organs and are also involved in the hemoglobin–NO cycle, which increases energy efficiency under hypoxia, by oxidizing NAD(P)H to enhance proton pumping and concomitant ATP production**.** At the 2014 XVIII Conference on Oxygen-Binding and Sensing Proteins, several prominent research groups focusing on heme proteins reached a consensus to rename these proteins as “Phytoglobins” (Hill *et al.,* 2016). In this context, *phyto* means plant (including algae and land plants) and *globin* refers to a heme-containing protein fold similar to the myoglobin structure of the sperm whale, where heme-Fe is

invariably coordinated at the proximal site by His F8. Hence, in agreement with Hill *et al.,* (2016), we recommend the term 'Phytoglobin' when referring to non-symbiotic hemoglobins in plants. This terminology applies to hexacoordinated, non-symbiotic hemoglobin 1 / class 1 (Phytogb 1), pentacoordinated non-symbiotic hemoglobins 2 / class 2 (Phytogb2) and penta/hexacoordinated, non-symbiotic hemoglobin 3 / truncated hemoglobins (Phytogb3) (Hill *et al.,* 2012).

It has been common practice to describe the reaction between oxyhemoglobin and NO as a "dioxygenase" reaction or "dioxygenase" activity, including phytoglobin (1,2), ascribing enzyme function to hemoglobin. There is no evidence of any hemoglobin acting as an enzyme, except for flavohemoglobin (Gardner *et al.,*1998) which is a bifunctional protein with true enzyme activity.

Nomenclature of nitric oxide synthase-like activity in plants

Nitric oxide synthase (NOS) is the main enzymatic source for NO in metazoans. This enzyme catalyses the production of both NO and L-citrulline from L-arginine using two co-substrates (NADPH, oxygen) and several cofactors including two flavins (FMN, FAD), calmodulin and a pterin (tetrahydro-L-biopterin). In contrast to several algal species, land plants do not possess a typical NOS (Jeandroz *et al.,* 2016). However, several lines of evidence suggest that activity resembling that of a NOS is present in land plants (Reviewed in Corpas *et al.,* 2009) and the identification of the protein(s) catalysing this activity is a major goal (Del Castello *et al.,* 2019)

In aggregate, we therefore suggest employing the terms NOS-like activity or NOS-like enzyme when referring to this enzymatic process and to the corresponding unidentified enzyme(s). We advocate that these terms can be utilised to describe L-Arg-dependent activities, as the NOS measured in land plants requires L-arginine, NADPH, calcium and calmodulin, also essential prerequisites of mammalian NOS enzymes. It has been suggested that this plant activity could be a result of cooperation between separate proteins, which, when combined, biochemically resemble the NADPH:oxygen oxidoreductases of animal NOSs (Corpas and Barroso, 2017).

Suggested best practice for NO detection methods

Due to the rapid chemical reactions exhibited by the NO free radical with a wide range of biological targets, the detection and quantification of this molecule in plant samples is routinely difficult. Further, the current methods deployed differ in terms of both their selectivity and specificity (Vishwakarma *et al.,* 2019). Unfortunately, to date, there is no entirely satisfactory method for the quantification of NO. Each of the current methods has specific limitations. However, by employing best practice, reliable results can be obtained, enabling successful interpretation NO function (Gupta and Igamberdiev 2013). Thus, the available methods of NO measurement performed

carefully can typically provide accurate and robust results *in vitro*, using NO or chemical compounds including NO donors. However, there are significant limitations when these approaches are applied to complex biological matrices without proper validation and control assays.

Therefore, the given technique, whether direct or indirect, should be selected with caution, given that all the current methods have both advantages and disadvantages. Those relevant to plant samples include colorimetric assays (based on Griess reactions and oxyhemoglobin), fluorimetric assays using different diaminofluorescein (DAF) dyes, photo-acoustic laser detection, electron paramagnetic resonance (EPR) spectroscopy with a NO-specific spin trap and ozone chemiluminescence detection utilising a NO-specific electrode (Mur *et al*., 2011). Due to its NO-specificity, spin trap EPR is considered one of the most specific methods for demonstrating the unequivocal presence of NO. In plants, this method has been useful in detecting the presence of NO in both plant extracts and purified organelles (Maskall *et al.*, 1977; Caro and Puntarulo, 1999; Corpas *et al.,* 2004; Jasid *et al.,* 2006).

Chemiluminescence is best suited for measurement of emitted NO but in order to measure oxidized forms of this molecule, one has to employ indirect chemiluminescence, where samples should be injected with solutions such as vanadium chloride (VClIII) to reduce oxidized forms of NO. Recently, genetically encoded NO probes have been described (Eroglu *et al*., 2016; Calvo-Begueria *et al.,* 2018). Such proteins are an optimal tool for NO detection/quantification *in vivo* and have the potential to revolutionize the field of plant NO research. Calvo-Begueria and colleagues monitored NO production via formation of a nitrosyl–leghemoglobin complex (Lb2+NO), which can be detected by EPR spectroscopy. Further, Eroglu *et al.,* (2016) fused a bacteria-derived NO-binding domain adjacent to different fluorescent protein variants, enabling both direct observation and quantification of NO. These genetically encoded NO probes provide a specific real-time, read-out of cellular NO dynamics and, hence, potentially open a new era for NO bioimaging.

In addition to these methods, the application of microelectrodes is also an effective approach, for example, in tracking NO in the extracellular media of cell suspensions. These electrodes consist of a platinum/iridium (Pt/Ir) wire sealed in a glass or plastic capillary in which a thin film of nickel phthalocyanine is electrodeposited. An outermost layer constituted from Nafion and o-phenylenediamine increased the selectivity of the electrodes against possible interfering molecules (Griveau *et al*., 2016). This method is considered as one of the most specific methods in animals and has also been successfully used to measure NO production in plant cell suspensions (Besson-Bard *et al.,* 2008). With appropriate controls: NO deficient mutants such as *nia1 nia2, atnoa1* or NO scavenging lines, including those overexpressing *Pgb1* or S-nitrosoglutathione reductase (*GSNOR*) (Yun et al., 2011), one can accurately determine the endogenous NO level. Also, the application of NO donors or scavengers as controls, can function as key controls in the determination of NO.

DAF-based dyes

Fluorescence-based methods for the detection of NO and other RNS are commonly utilised (Mur *et al.,* 2011). The technique relies on the presence of a non-fluorescent probe which can be located to the source of NO and subsequently becomes fluorescent on reaction with this molecule or a related reactive nitrogen species (RNS). Therefore, a major advantage to this approach is that it can provide spatial information regarding the accumulation of the specific RNS under study.

There are however, numerous potential problems with this approach and often such issues are not considered. The technique replies on the measurement of fluorescent light, which does not readily lend itself to quantitation and is thus usually reported as pixel intensity, not molarity. Therefore, measured light relies on efficient penetration of the excitation light and efficient release of emitted light, both which can be problematic within deeper samples. This approach can also be affected by autofluorescence of the sample, relatively common in plant material and is also prone to photobleaching. It also noteworthy that RNS accumulation is typically not static, but can be repositioned, as reported for peroxynitrite ($ONOO^-$) moving through membranes (Denicola *et al.*, 1998). Created fluorescent RNS-adducts can also move, so spatial data is not always reliable.

However, one of the biggest concerns regarding DAF-based dyes is specificity and selectivity. Fluorescent probes rely on redox chemistry and it would not be unusual for such probes to be oxidised by a range of endogenous redox active molecules. For example, 2,7-dichlorodihydrofluorescein (DCFH) oxidation yields 2,7-dichlorofluorescein (DCF), a reaction which can be driven by the presence of RNS but also reactive oxygen species (ROS). Indeed, DCFH is commonly utilised to detect ROS. Issues and limitations of the fluorescent-based approach to RNS measurements have been the subject of several reviews (Kalyanaraman *et al*., 2012; Li and Wan, 2015).

However, despite these potential limitations, the application of these reporter dyes can often be effective. The most commonly used probes are based on diaminofluorescein (DAF): i.e. DAF-2DA and DAF-FM DA, both readily commercially available. On entering the cell, intracellular esterases cleave these dyes to DAF-2D or DAF-DM and subsequently, react with RNS leading to the formation of a nitrosated form, DAF-2 triazole (DAT-2T), which is fluorescent. Importantly, however, DAF does not react directly with NO. It reacts with oxidized forms such as NO^+ or N_2O_3. Although DAF is relatively specific to RNS, DAF-2DA can also react with ROS (Balcerczyk *et al*., 2005). Hence, it is essential to check the specificity of fluorescence employing an NO scavenger, to confirm the detected fluorescence is resulting from NO accumulation, as ROS and NO are often produced in parallel. An ideal NO scavenger to utilise in this case is 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (cPTIO, see below). It should be also considered that DAF-2 itself is weakly fluorescent, so in some instances the observed increased fluorescence can result from its accumulation inside cells and not by its reaction with RNS. This potential scenario can be checked by using 4-aminofluorescein DA (which is converted to 4-AF), as a negative control, which cannot react with NO (Beligni *et al.,* 2002).

A variation of this approach can also be employed to measure exogenous or released RNS. DAF2-DA is not fluorescent, but if the de-esterified version is used it is unable to penetrate cells but can react with RNS and become fluorescent and so extracellular RNS can be estimated. Such measurements can then be corroborated by more difficult approaches, including EPR.

As well as DAF-based probes, there are other fluorescent dyes available for RNS measurements, such as the copper (II) fluorescein complex (CuFL) (Lim *et al.,* 2007), the diaminorhodamine-4M probes (DAR-4M, Kojima *et al.,* 2001), or the Pyrene-Based Fluorescent Nitric Oxide Cheletropic Traps (FNOCTs, Düppe et al., 2010). CuFL has the advantage of reacting directly with NO itself rather than a derivative RNS and is an interesting alternative to that of DAF. For the measurement of peroxynitrite, dihydrorhodamine 123 (DHR) can be employed as it yields the fluorescent compound rhodamine 123 (RH) on oxidation. There are numerous other fluorescent probes, such as those based on aromatic boronates (Kalyanaraman *et al*., 2012). The application of one of these dyes for RNS detection as the sole method is not recommended and other techniques should be employed in parallel to ensure robustness of RNS data (Gupta and Igamberdiev 2011).

NO scavenger controls

cPTIO is a widely used NO scavenger to confirm any observed DAF fluorescence is attributed to NO. cPTIO oxidizes the NO molecule leading to formation of the NO_2 radical (NO + cPTIO → NO_2 + cPTI). The produced NO_2 radical can react with NO to form N_2O_3 (NO_2 + NO → N_2O_3) with which DAF-2 reacts leading to formation of fluorescent DAF-2T (Table 1). This implies, to some extent, cPTIO has the capability to increase fluorescence rather than masking it, if used at a low concentration. At a high concentration, cPTIO reacts rapidly with NO and oxidizes NO to NO_2+PTI. Hence, a concentration of > 200 µM of CPTIO is recommended (Vitecek 2008). Nevertheless, cPTIO is also known to quench DAF-2T fluorescence due to its intense blue colour (Arita *et al.,* 2006). Thus, the optimization of cPTIO concentrations for any given experimental setting is highly advised (Goldstein *et al.,* 2003; D´Alessandro *et al.,* 2013). It is noteworthy that the reaction product, cPTI, has been reported to possess biological activity without NO scavenging, both in animal and plant models (Cao *et al.,* 2002; Planchet *et al.*, 2006), pointing to cautious interpretation of data obtained using DAF-based dyes and PTIO compounds. cPTIO can also be used as a spin trap in EPR to detect NO; however, this approach has also been shown to have considerable limitations (D´Allesandro *et al.,* 2013). Recently, it has been demonstrated that commercially available hemoglobins can be used as a control instead of cPTIO for attributing the fluorescence based MnIP-Cu probes specificity to NO (Singh and Bhatla 2019).

Collectively, therefore careful consideration should be given before embarking on experiments employing NO scavengers.

Application of NO donors

Treatment of plants with gaseous NO requires special equipment and special care to prevent gas leakage, so the application of NO releasing chemicals (NO donors) provides a more easily executable way of NO treatment. Therefore, supplying plants with different NO donors is a common practice to mimic NO production and potentially rescue NO deficient phenotypes. Different NO donors have different kinetics, mechanisms and environmental conditions for optimal NO release, thus some care should be taken during the choice of NO donor.

In plant research, the most commonly deployed NO donors are sodium nitropusside (SNP), S-nitrosopenicillamine (SNAP), S-nitrosoglutathione (GSNO) and diethylamine NONOate (DETA/NO). One should note that these donors differ in the form of NO release. For instance, SNP releases nitrosonium cation (NO^+) whereas SNAP and GSNO typically release NO in the form of a radical ($^{\cdot}NO$), but under certain environmental conditions these NO donors can also release the nitrosonium cation (NO^+). Accumulating evidence suggest that the form of NO emitted by various donors plays a key role in switching on appropriate metabolic modifications (Arasimowicz-Jelonek *et al.,* 2011).

Another relevant difference between donors is the kinetics of NO release. In aqueous solution, DEA/NO and SNAP produce transient NO bursts (seconds to minutes), while the NO-releasing effect of SNP is more extended (Planchet and Kaiser, 2006; Floryszak-Wieczorek *et al.,* 2006; Mur *et al.,* 2013). In a similar fashion, GSNO also delivers NO over a longer time period, typically several hours (Floryszak-Wieczorek *et al.,* 2006; Mur *et al.,* 2013). SNP is among the most widely studied NO donors, which is justified by its capability of producing persistent NO (Mur *et al.,* 2013) and by its cost-efficiency. However, the application of SNP has several drawbacks. Firstly, the release of NO requires light and the illumination may influence plant samples in an unwanted way. Secondly, NO release from SNP is associated with the production of toxic gases like hydrogen cyanide (HCN) (Table 1) (Bethke *et al.,* 2006). This compound can inhibit molybdenum based enzymes and also can inhibit cytochrome c oxidase leading to inhibition of respiration. However, Shishido & Ganzarolli de Oliveira (2001) reported that SNP releases CN following ultra-violet (UV) radiation, while illumination with longer wavelengths results in selective NO release from SNP, which supports the utility of this NO donor under typical light conditions. Reflecting these differences between NO donors, these compounds may have different biological effects: SNP induced the accumulation of *Ferritin* transcripts, while SNAP inhibited the expression of this gene. Similarly, SNP induced cell death and inhibited antioxidant gene expression but other NO donors showed opposing effects (Murgia *et al.,* 2004).

Furthermore, the NO releasing capacity of various donors depends on cellular redox and antioxidant status (Floryszak-Wieczorek *et al.,* 2006) and also on the actual concentration of the NO donor applied (Ederli *et al.,* 2009). Development of precise NO releasing compounds is an important task for future NO research. Until this has

been accomplished, it is recommended to test different concentrations of NO donors to determine their NO releasing effects *in vitro* and *in vivo* under the same experimental conditions. Appropriate control treatments (e.g. potassium cyanide, reduced glutathione) are also needed in order to support the NO releasing capacity of the different NO donors. The application of an NO scavenger (e.g. cPTIO) together with an NO donor can provide useful information regarding the NO releasing character of the donor.

Another experimental option is subjecting the biological system under study to NO gas (Palma *et al.,* 2018). Currently, the NO scientific community is searching for "elicitors" which promote endogenous NO release, enabling more physiological responses. The development of either genetically encoded or chemically based, organelle specific NO reporters, would be also an important future advance.

Enzyme inhibitors of NO metabolism

In the context of the pharmacological approach, several types of compounds have been employed to study the involvement of specific plant enzyme(s) in NO production or signalling pathways. This extended practice might result in hard to interpret data, due to known or unknown unspecific effect of these compounds to other plant proteins or enzymes and partly also due to their application in relatively high concentrations, often required to achieve any observable effects.

A good example of this is tungstate, which can inhibit nitrate reductase (NR) activity through molybdenum displacement and has been used to confirm involvement of NR in observed NO production (Chamizo-Ampudia *et al.,* 2017**)**. However, tungstate is known to interfere with other molybdenum-containing enzymes and also plant developmental processes (Xiong *et al.,* 2012). As tungstate is known to affect both plant NR activity and gene expression (Deng *et al.,* 1989), experiments using tungstate to test NR-dependent NO production should also involve determination of NR activity.

A high number of plant studies have employed chemical substances developed as effective inhibitors of well-characterized animal NOS isoforms. This practice, based on diverse L-arginine derivatives such as (N^G-monomethyl-L-arginine, L-NMMA and N-$^{\omega}$-nitro-L-arginine, L-NAME), has been a subject of long-term criticism (Planchet and Kaiser 2006). This is mainly for two reasons: the application of high concentrations of these compounds (orders of magnitude higher compared to animal NOS studies) and possible inhibitory effects on other plant enzymes, such as arginase or arginine decarboxylase (Reisser 2002), iron-containing enzyme (Peterson *et al.,* 1992) and NR (Rasul *et al*., 2012). Therefore, as standard good experimental practice, the use of L-arginine derivatives should also include their inactive D-enantiomers as a control. However, as their true molecular targets in land plants still remain enigmatic, corresponding caution is advised in the interpretation of results derived from the application of these NOS inhibitors.

Conclusions

It is apparent that significant methodological improvements are required in plant NO research to support more robust data acquisition. The plant research community should also be open to the adaption of methods and approaches from animal studies, but these should be applied with care. In the meantime, the existing procedures and methods should be deployed in a careful and thoughtful fashion to mitigate their disadvantages, following whenever possible the recommendations as summarized below:

- NO/RNS detection and/or quantification should include at least two different methods based on different principle/reaction mechanisms.

- Application of a pharmacological approach, such as chemical NO/RNS donors, scavengers or inhibitors, should include available negative and/or positive controls in a range of concentrations.

- Methods transferred directly from the animal NO field, should be subjected to careful testing and validation of their applicability on specific plant species.

- Data interpretation should take account of known methodological limitations, including possible unspecific reactions and interference by ROS and other plant reactive compounds. The detection limits of the employed methods and their non-quantitative nature in certain experimental settings should also be considered.

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Table 1: Overview of commonly used NO donors, reactants, detection reagents and their reactions

Compound	Chemical name	Reaction(s)	Comment
NO donors			
Nitrite	NO_2^-	$NO_2^- + e^- + 2H^+ \rightarrow NO + H_2O$	Rate of NO release is highly pH-dependent
SNP	Sodium nitroprusside	$Na_2[Fe(CN)_5NO]^{2-} \rightarrow NO^{\cdot} + CN^- + Na_2[Fe(CN)_4]^-$ $Na_2[Fe(CN)_5NO]^{2-} \rightarrow NO^+ + CN^{\cdot} + Na_2[Fe(CN)_4]^-$	Light-dependent reaction, SNP can also release nitrosonium and free iron
GSNO	S-nitrosoglutathione	2 GSNO $\rightarrow$ 2 $NO^{\cdot}$ + GSSG (decomposition) GSNO + RSH $\rightarrow$ RSNO + GSH (transnitrosation)	Reaction catalyzed by light, heat and metal ions (Cu^{2+}, Hg^{2+}), under certain conditions also nitrosonium (NO^+) can be formed
SNAP (and other S-nitrosothiols)	S-Nitroso-N-acetyl-DL-penicillamine	2 RSNO $\rightarrow$ 2 $NO^{\cdot}$ + RSSR	Reaction catalyzed by light, heat and metal ions (Cu^{2+}, Hg^{2+}), under certain conditions also nitrosonium (NO^+) can be formed
DEA NONOate (and other NO-amine adducts)	Diethylamine NONOate	$R_2N\text{-}NO\text{-}NO + H^+ \rightarrow 2\ NO^{\cdot} + R_2\text{-}NH_2$	Rate of NO release from NONOate is highly pH-dependent
Peroxynitrite donors			
SIN-1	3-Morpholinosydnonimine	SIN-1 $\rightarrow$ $NO^{\cdot} + O_2^-$ + SIN-1C $\rightarrow$ $ONOO^-$ + SIN-1C	Spontaneous decomposition in presence of oxygen
NO reactions in biological millieu			
Oxygen	O_2	NO + O_2 $\rightarrow$ NO_2	End-products: NO_2^-, NO_3^- (in presence of hemoglobins)
Superoxide anionradical	$O_2^{-\cdot}$	NO + $O_2^{\cdot -}$ $\rightarrow$ ONOO-	End-products: NO_3^-
Thiols	R-SH	NO + O_2 $\rightarrow$ NO_2 + NO $\rightarrow$ N_2O_3 N_2O_3+ RSH $\rightarrow$ RSNO + NO_2^- + H^+	End-products: S-nitrosothiols, nitrite, disulfides or mixed sulfides

Oxyphytoglobins	$HbFe^{2+}O_2$	$HbFe^{2+}O_2NO$	End-products: NO_3^-, metaphytoglobin,
NO detection			
Reaction partner	**Chemical name**	**Reaction(s)**	**Comments**
O_3	Ozone	$NO + O_3 \rightarrow NO_2^*$ $NO_2^* \rightarrow NO_2$ + light	Reaction exploited in specialized instruments such as a chemiluminiscence detector for analysis of NO, nitrites and S-nitrosothiols
cPTIO	(2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt)	$NO^\cdot$ + cPTIO $\rightarrow NO_2^\cdot$ + cPTI	Carboxy derivative (cPTIO) is preferably used as NO scavenging controls due to higher pH stability
DAF-2 / DAF-2 DA	4,5-diaminofluorescein diacetate	$NO + O_2 \rightarrow NO_2$ $NO_2^\cdot$ + DAF-2 $\rightarrow$ DAF-2T	Reaction of NO with difluorescein-based probes is O_2-dependent
DAF-FM/DAF-FM DA	4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate	$NO + O_2 \rightarrow NO_2$ $NO_2^\cdot$ + DAF-FM $\rightarrow$ DAF FM- 2T	Reaction of NO with DAF FM probes is O_2- and pH dependent